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Effects of Anticoagulants on Lipoprotein(a) Measurements with Four Commercial Assays

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Summary: Lipoprotein(a) levels in plasma are considered an independent risk factor for atherosclerosis at different sites. Although Lp(a) measurements have recently gained interest in clinical laboratories, several problems are still unresolved.

A potential source of pre-analytical variability lies in the treatment of the specimens, since it has been reported that values of several lipid quantities are lower when measured in plasma instead of serum.

Lp(a) was measured in serum and in EDTA-treated, heparinised and citrated plasma from 15 healthy volunteers. Four analytical methods were used: two enzyme linked immunosorbent assays [ELISA] based on a polyclonal anti-apolipoprotein(a) antibody and a polyclonal anti-apolipoprotein B antibody, respectively; and two immunonephelometric assays [INA] based on a N antiserum to Lp(a) and on three monoclonal antibodies adsorbed on latex particles, respectively.

Our measured Lp(a) values in plasma were lower than those found in serum, in particular for EDTA-treated (anti-apolipoprotein(a) ELISA: $p < 0.01$, anti-apolipoprotein B ELISA: $p < 0.001$ and Latex enhanced INA: $p < 0.001$) and citrated plasma (anti-apolipoprotein(a) ELISA: $p < 0.05$, anti-apolipoprotein B ELISA: $p < 0.001$ and INA: $p < 0.001$). Lp(a) values measured in heparinised plasma were also lower than those found in serum, but the difference was not statistically significant.

Introduction

Lipoprotein(a) [Lp(a)] is a cholesterol-rich lipoprotein in which the polymorphic glycoprotein apolipoprotein(a) is covalently linked to apolipoprotein B 100, the main protein moiety of LDL (1).

Apolipoprotein(a) structure is closely related to plasminogen; its size polymorphism results from multiple repeats of the plasminogen-like kringle IV domain, which give rise to 34 or more isoforms in plasma (2).

Since several studies have reported a strong correlation between increased levels of Lp(a) in plasma and both clinical and preclinical atherosclerosis (3–6), Lp(a) measurements have recently gained popularity in clinical laboratories.

The main problem in Lp(a) measurements arises from the lack of a primary standard for secondary calibration of the assays employed in Lp(a) analysis. Moreover, due to the apolipoprotein(a) size polymorphism, the use of polyclonal antibodies in immunometric commercial kits recognising different apolipoprotein(a) domains often results in discordant data among assays.

EDTA-plasma or serum are the preferred specimens for Lp(a) assays but little is known about the effect of anticoagulants on Lp(a) measurements. *Cooper* et al. considered EDTA as the preferred anticoagulant for routine analysis of lipoproteins and lipids since it is regarded as more efficient both in preventing lipids oxidation and in preserving the immunoreactivity of the particles (7). On the other hand, it has been reported that values for total cholesterol, triacylglycerols and high density lipoprotein-cholesterol, as measured with several analytical methods, are significantly lower in plasma than in serum (7, 8).

It has also been observed that Lp(a) values measured in plasma are different from those found in serum; this hinders the definition of the normal range of concentrations among laboratories and comparisons in epidemiological studies.

The aim of the present study was to compare results of Lp(a) measurements in serum and plasma (EDTA-treated, citrated and heparinised) obtained with four commercial kits.

Materials and Methods

Plasma samples

Whole blood from 15 healthy volunteers was collected after one night fasting in 8 ml evacuated tubes containing either no anticoag-

ulant or alternatively disodium EDTA (final concentration 4.46 mmol/l), lithium-heparin (final concentration 143 IU/l), buffered sodium citrate (final concentration 0.129 mol/l). Serum and plasma were removed from cellular components by centrifuging at 3000 min^{-1} for 20 min. Samples were analysed within 2 h after venipuncture. Results obtained in EDTA and citrated plasma were adjusted for dilution.

Lp(a) concentration was measured with two enzyme linked immunosorbent assays [ELISA] and with two immunonephelometric assays [INA].

Enzyme immunoassays

The first ELISA method (Macra Lp(a) – Terumo, Elkton, Ma, USA) uses monoclonal anti-apolipoprotein(a) antibody coated plates for capturing the Lp(a) particles and a second horseradish peroxidase-conjugated anti-apolipoprotein(a) polyclonal antibody for the detection.

All the samples were analysed in duplicate and the average value was reported. Coefficients of variation (CVs) were respectively 3% within-assay and 5% between-assay.

The second ELISA method (Innotest Lp(a) – Byk-Sangtec Diagnostica, Dietzenbach, Germany) uses monoclonal anti-apolipoprotein(a) antibody coated plates for capturing the Lp(a) particles and a second horseradish peroxidase-conjugated polyclonal anti-apolipoprotein B antibody for the detection. All the samples were analysed in duplicate and the average value was reported. CVs were respectively 4% within-assay and 5% between-assay.

Although the standard curves of both the assays range respectively from 0 to 800 mg/l and from 0 to 1000 mg/l of Lp(a) concentration, we arbitrarily chose dilutions of each sample in order to obtain absorbance values in the middle part of the standard curve, thus reducing the bias effect of high and low absorbances.

Immunonephelometric assays

The first INA was performed on a Behring Nephelometric Analyser (BNA, Behringwerke, AG, Marburg, Germany) using a N antiserum to human Lp(a). Results were evaluated by means of logit-log function of light scattering intensities vs. respective concentrations of scalar dilutions of Lp(a) standard (N Lp(a) Standard – Behring). The assay involves 1 : 5 dilution of the sample; each aliquot was analysed in triplicate and the average value was reported.

The second INA was performed on BNA using three monoclonal antibodies adsorbed on latex particles. Results were evaluated by means of logit-log function of light scattering intensities vs. respective concentrations of scalar dilutions of Lp(a) standard (N Lp(a) Standard – Behring). The assay involves 1 : 100 dilution of the sample; each aliquot was analysed in duplicate and the average value was reported.

All results were reported as Lp(a) total mass (mg/l).

Statistical evaluation

Non-parametric correlation was done according to *Passing & Bablok*. Significance was calculated using *Wilcoxon*'s matched-pairs signed ranks test, and regression was performed according to *Spearman*.

Results

Anti-apolipoprotein(a) ELISA

Lp(a) values measured in anticoagulant-treated plasma tend to be lower than those measured in serum (fig. 1). In particular, results from EDTA and citrated plasma (but not heparin-plasma) are significantly lower ($p < 0.01$ and $p < 0.05$ respectively) than those from serum (tab. 1). Correlation coefficients between serum and plasma are $r = 0.995$ for EDTA-treated, $r = 0.986$ for heparinised and $r = 0.996$ for citrated plasma, respectively.

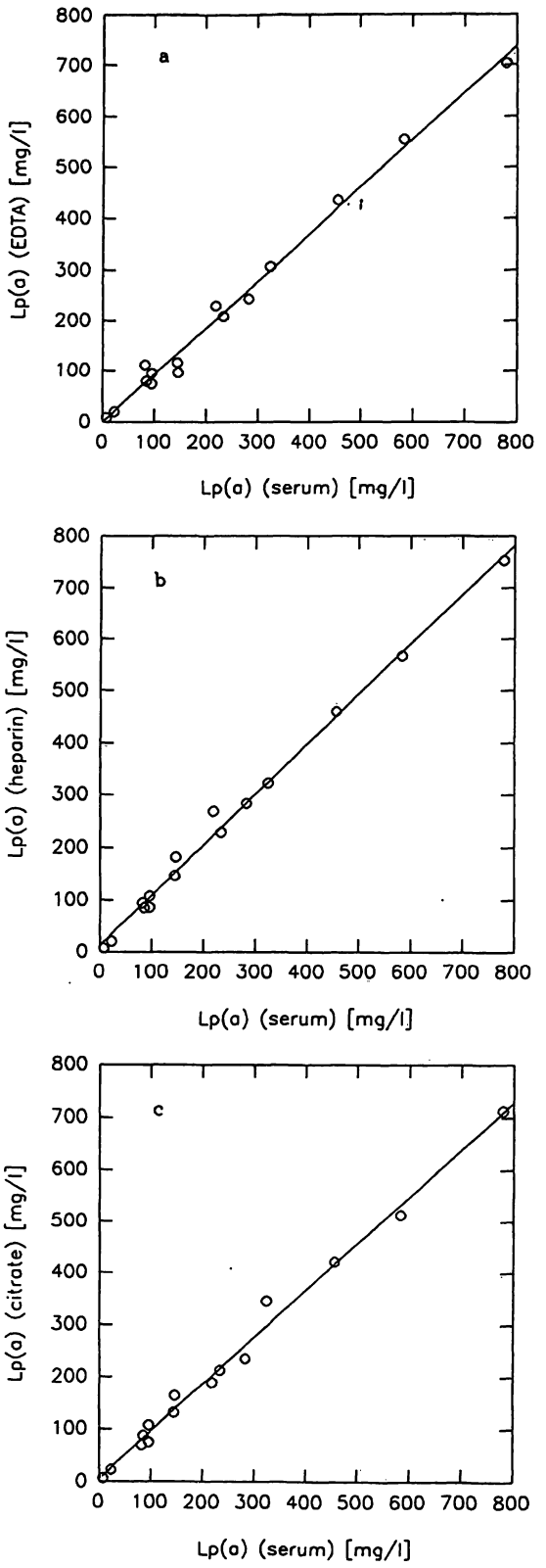

Fig. 1 Comparison of Lp(a) concentrations in serum and in EDTA-treated, heparinised and citrated plasma, measured with an ELISA employing a polyclonal anti-apolipoprotein(a) antibody. Non-parametric properties of the curve and correlation coefficients according to *Passing & Bablok* are:
$y = 0.928x - 0.271$ and $r = 0.995$ (a),
$y = 0.983x + 4.96$ and $r = 0.986$ (b),
$y = 0.912x + 0.05$ and $r = 0.996$ (c).

Tab. 1 Statistical significance of Lp(a) concentrations determined with four analytical methods in serum and in EDTA-treated, heparinised and citrated plasma, using *Wilcoxon*'s matched-pairs signed ranks test.

	Anti-apolipoprotein(a)	Anti-apolipoprotein B	INA	Latex − INA
Serum/ EDTA	$p < 0.01$	$p < 0.001$	n.s.	$p < 0.001$
Serum/ Heparin	n.s.	n.s.	n.s.	n.s.
Serum/ Citrate	$p < 0.05$	$p < 0.001$	$p < 0.001$	n.s.

Anti-apolipoprotein B ELISA

As shown in figure 2, Lp(a) values tend to be lower in anticoagulated plasma than in serum. In particular results from EDTA and citrated plasma (but not heparin-plasma) are significantly lower ($p < 0.001$ and $p < 0.001$ respectively) than those from serum (tab. 1). Correlation coefficients between serum and plasma are $r = 0.996$ for EDTA-treated, $r = 0.996$ for heparinised and $r = 0.986$ for citrated plasma, respectively.

Immunonephelometry

As shown in figure 3, only citrated plasma shows lower Lp(a) values compared with those in serum ($p < 0.001$) (tab. 1). Correlation coefficients between serum and plasma are $r = 0.986$ for EDTA-treated, $r = 1.000$ for heparinised and $r = 0.994$ for citrated plasma, respectively.

Latex-enhanced immunonephelometry

As shown in figure 4, only EDTA plasma shows lower Lp(a) values compared with those in serum ($p < 0.001$) (tab. 1). Correlation coefficients between serum and plasma are $r = 0.996$ for EDTA-treated, $r = 0.986$ for heparinised and $r = 0.989$ for citrated plasma, respectively.

Discussion

Lp(a) measurements have gained interest due to the repeatedly reported correlation between increased Lp(a) concentrations in plasma and atherothrombotic diseases (3–6).

The collection and treatment of specimens represent a major source of inter-laboratory variation. It has been reported that values of several lipids and lipoproteins analysed with different methods are lower in EDTA and citrated plasma than in serum (7, 8). The differences persist after correction for dilution factors, and it has been suggested that they may arise from a shift of water from blood cells into plasma induced by the anticoagulants.

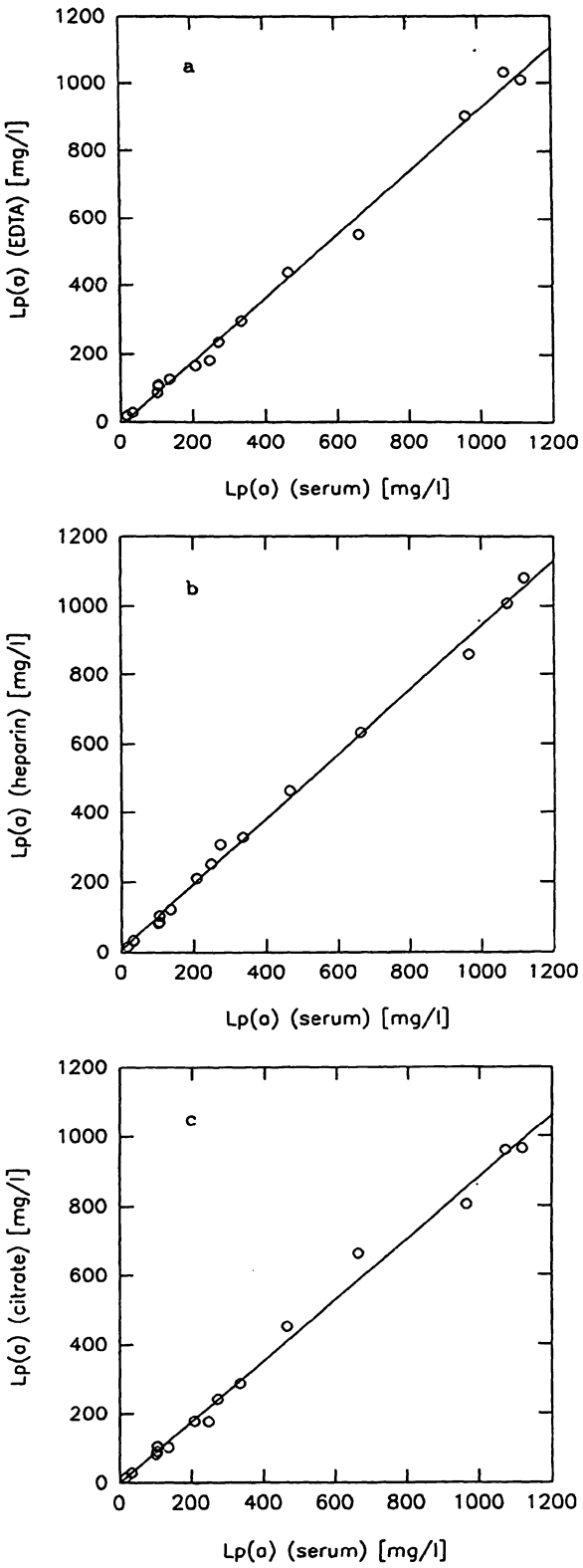

Fig. 2 Comparison of Lp(a) concentrations in serum and in EDTA-treated, heparinised and citrated plasma, measured with an ELISA employing a polyclonal anti-apolipoprotein B antibody. Non-parametric properties of the curve and correlation coefficients according to *Passing & Bablok* are:
$y = 0.916x - 2.554$ and $r = 0.996$ (a),
$y = 0.973x - 0.51$ and $r = 0.996$ (b),
$y = 0.874x + 0.86$ and $r = 0.986$ (c).

In our study we compared the effects of three widely used anticoagulants on Lp(a) measurements with four commercial Lp(a) assays.

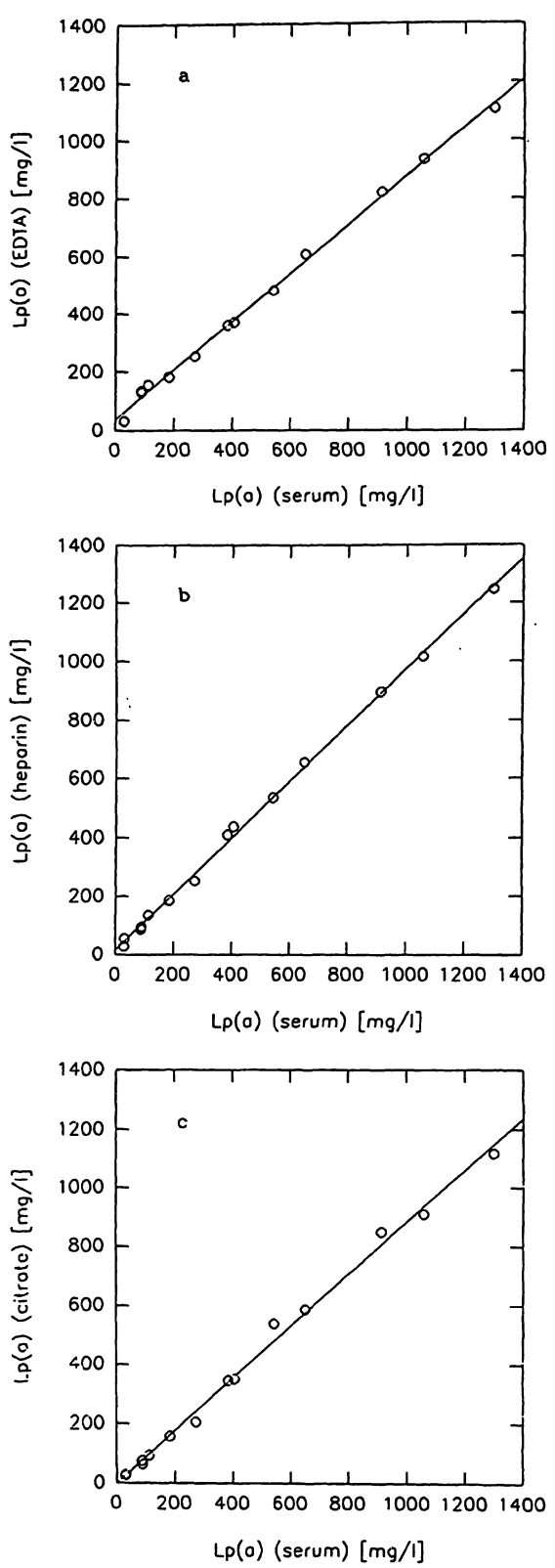

Fig. 3 Comparison of Lp(a) concentrations in serum and in EDTA-treated, heparinised and citrated plasma, measured with an INA method employing a N antiserum to human Lp(a). Non-parametric properties of the curve and correlation coefficients according to *Passing & Bablok* are:
y = 0.843x + 35.52 and r = 0.986 (a),
y = 0.966x + 8.51 and r = 1.00 (b),
y = 0.869x + 0.66 and r = 0.994 (c).

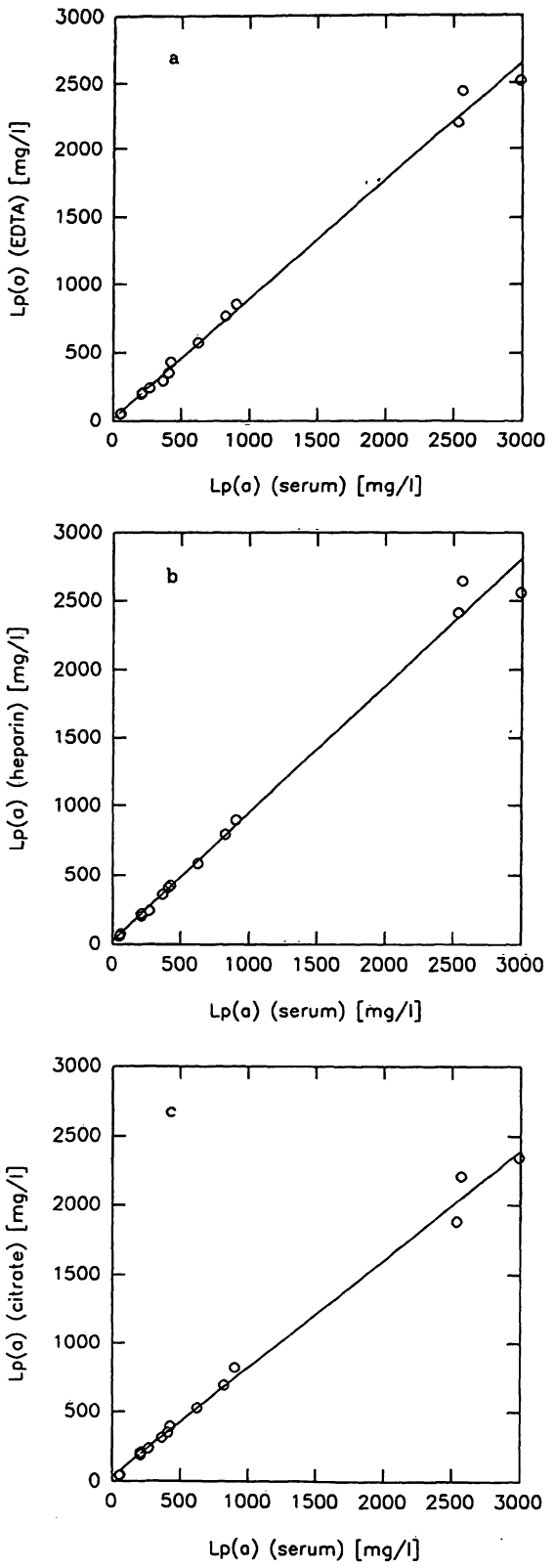

Fig. 4 Comparison of Lp(a) concentrations in serum and in EDTA-treated, heparinised and citrated plasma, measured with a latex-enhanced INA method employing three monoclonal antibodies adsorbed on latex particles. Non-parametric properties of the curve and correlation coefficients according to *Passing & Bablok* are:
y = 0.916x + 1.826 and r = 0.996 (a),
y = 0.957x + 6.22 and r = 0.986 (b),
y = 0.834x + 10.90 and r = 0.989 (c).

Correlation coefficients between serum and plasma were always good, and the assays always within the limits of analytical variability.

In spite of the adjustment for dilution, plasma Lp(a) values always tended to be lower than those in serum. In particular, values in EDTA-treated and citrated plasma

were significantly lower (figs. 1, 2, 3 and 4) in three of the four assays (tab. 1). Values in heparinised plasma were also lower than in serum (figs. 1, 2, 3 and 4) but the difference was not statistically significant (tab. 1). It should be noted that the decrease of plasma Lp(a) immunoreactivity in serum seems to parallel the progressive decrease of the anticoagulant concentration in the sample, tending to be higher for citrated plasma (129 mmol/l final concentration), intermediate for EDTA plasma (4.46 mmol/l final concentration) and lower for heparinised plasma (1.2 mmol/l final concentration). A similar effect was observed after analysing apolipoprotein A-I and apolipoprotein B in serum, heparinised and EDTA-treated plasma from the same patients (unpublished data). These observations may support the proposal that water moves osmotically from the blood cells into the plasma.

General agreement is still lacking concerning certain theoretical and methodological aspects of the standardization of lipoprotein(a) measurements. In this respect, the exclusion of any possible source of pre-analytical variability is a prerequisite to obtaining comparable results between laboratories employing different methods.

In our study Lp(a) values in plasma were generally lower than those in serum; since agreement among methods is satisfactory, as deduced by the correlation coefficients, the effect of dilution can be safely forecast.

Moreover, when employing anticoagulants, the laboratory must be informed about the type of specimen in order to consider a possible correction of results.

On the basis of the above precautions, we suggest that serum or anticoagulant-treated plasma may both be used in Lp(a) analysis.

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